Studies on the Phytochemical and Hepatoprotective Activity of the medicinal plants- Lagenandra toxicaria and Ariopsis peltata

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Chapter One

Introduction

1.1 Introduction

From prehistoric times plants have been utilized in traditional medicine and as nutrition. Chemical constituents and secondary metabolites in the plants are unique to each plant. Over the years, knowledge on medicinal plants has been gathered, which lead to the development of several medical systems such as Ayurveda, Unani, and Siddha. Traditional healers in India employ around 2500 plant species, with about 100 species serving as a regular supply of medication (Muthu et al., 2006; Sheng-Ji, 2001). Secondary metabolites of medicinal plants, such as flavonoids, phenolics, terpenoids, alkaloids, and sulphur-containing compounds have been successfully used in medicines and bioactive products. In this study, we chose two medicinal plants that belong to the family Araceae. It is the most diverse family in the monocotyledons. Several species of *Araceae* members are used for food and medicinal

purposes. About 10% of the world's population consumes taro corms (Frausin et al., 2015). Many species of *Araceae* are used to treat malaria and liver disease symptoms in traditional knowledge. The plant *Acorus calamus* is used as carminative, antidiarrheal in China and India. The components, especially α and β-asarone have a wide variety of pharmacological properties including CNS depressant, behaviour altering, anti-inflammatory, sedative and anticonvulsant (Mukherjee et al., 2007). *Amorphophallus konjac* is used to treat wounds, tumours, and skin diseases; this plant tuber is also used as a food source in tropical Asia (Chua et al., 2010). Many species of *Araceae* show antibacterial activity and antifungal activity, such as *Amorphophallus bequaerii, Sauromatum guttatum,* and *Syngonium podophyllum* (Chua et al., 2010; Said et al., 2019). Aroids are among the first plants used to fight cancer. *Pinellia ternata*, commonly known as 'Ban Xia' in Chinese, was known to be used in the first century B.C. for the treatment of breast and stomach cancer and leukaemia (Chen et al., 2007).

Lagenandra toxicaria Dalz

The herb, *Lagenandra toxicaria* is exclusively found in southern India, and it is commonly seen in marshy areas. The plant's rhizome is used in traditional medicine to cure bilious symptoms, as well as renal and cardiac illnesses.

Ariopsis peltata.

Ariopsis peltata is a small perennial herb. It is a lithophytic, tuberous herb; tubers of this plant grow almost up to 1-5 cm radius. Mature plants grow up to 15-25 cm long and can be found distributed abundantly across the Indian plains, western ghats, and from Nepal to Sikkim. *Ariopsis peltata* mainly grows in the monsoon season, and it's used as a food source in some regions, mainly the leaves.

In spite of their known usage in traditional medicine and utilization of plant-derived products in modern therapeutics, very little has been studied about the phytochemical and pharmacological activities of these two plants. Hence, this study is intended to carry out certain phytochemical and pharmacological studies on these two plants.

1.2 Objectives:

1. phytochemical analysis. *in-vitro* antioxidant and *in-vitro* antidiabetic studies.
2. Nutritional, anti-nutritional component analysis, isolation and partial characterization of starch.
3. *In-vivo* genotoxic and antigenotoxic analysis.
4. Hepatoprotective activity of the plant extracts and identification of the bioactive compound.

Chapter Two

Phytochemical analysis and *In-vitro* antioxidant and antidiabetic activity of *Lagenandra toxicaria* Dalz and *Ariopsis peltata* rhizome extracts.

2.1 Introduction

The living cells generate free radicals and other reactive oxygen species by-products as a result of physiological and biochemical processes. Free radicals cause oxidative damage to DNA, proteins and lipids, eventually leading to many chronic diseases, such as cancer, diabetes, ageing, and other degenerative diseases in humans (Lobo et al., 2010; Pham-Huy et al., 2008). Plants are a well-known source of several potent antioxidants like polyphenols, flavonoids, isoflavonoids, anthocyanidins, phytoestrogens, terpenoids, carotenoids, limonoids, phytosterols, glucosinolates, and fibres are among the phytochemicals described as having possible health advantages. Previous reports have shown that many of these antioxidant compounds have been found to have anti-inflammatory, anti-fibrotic, anticancer, antimutagenic, antigenotoxic and antibacterial, properties (Thakur et al., 2020). Since plants are known to be the source for many drugs, extended scientific investigation on previously unexplored plants for their bioactive components is required.

2.2 Methods

Lagenandra toxicaria (LT) and *Ariopsis peltata* (AP) rhizomes were collected from Markanja village, Sullia taluk (12º34'38.7"N, 75º.29'54.8"E) and Charmadi ghat, Belthangadi taluk (13º04'35"N, 75º27'09"E) respectively. The plant rhizomes were washed, dried and powdered. The dried rhizome powder was extracted in petroleum ether, ethyl acetate, and methanol using the soxhlet apparatus. The aqueous extract was prepared by using the water bath. The extract was dried under vacuum at 45ºC and stored at 4ºC for further analysis. The yield of each extract was expressed in percentage. The qualitative analysis of phenol, flavonoid, tannin, saponin, alkaloid and steroid was carried out following Harborne (1998). The quantitative estimation of the polyphenols, flavonoids and tannins was done using the spectrophotometric method (Sadasivam, 1996). *In-vitro* antioxidant properties were analyzed using DPPH and reducing power assay and IC 50 of the plant extract was determined. The *in-vitro* antidiabetic activity was examined using inhibition of α-amylase and inhibition of α-glucosidase enzyme activity following Al-Hajj et al., (2016).

2.3 Results

2.3.1 Yield of extracts

Both the *Lagenandra toxicaria* and *Ariopsis peltata* showed a high yield in methanol and water compared to the petroleum ether and ethyl acetate.

2.3.2 Phytochemicals analysis

The preliminary analysis of LT and AP methanol and water extracts revealed the presence of phenols, flavonoids, tannin and saponin. Alkaloids were absent in the extracts. Steroids were present in all the extracts except water extract of both the LT and AP.

2.3.3 Quantitative analysis

2.3.3.1 Determination of total phenolic content.

In the total polyphenolic estimation, the *Lagenandra toxicaria* methanol extract showed more polyphenols compared to the other extracts.

2.3.3.2 Estimation of flavonoids.

In the total flavonoid estimation, the *Lagenandra toxicaria* methanol extract showed the presence of high amount of flavonoid compare to the other extracts.

2.3.3.3 Estimation of Tannin.

Ariopsis peltata methanol extract showed the presence of higher content of tannin compared to the other extracts.

2.3.4 *In-vitro* antioxidant activity assay

2.3.4.1 DPPH antioxidant assay: - The test revealed that the methanol extract of *Lagenandra toxicaria* has better antioxidant activity compared to the other extracts

2.3.4.2 The reducing power assay: - *Lagenandra toxicaria* methanol extract showed higher absorbance in 1000 µg/ml compared to the other extracts.

2.3.5 Antidiabetic study

2.3.5.1 *In-vitro* α-amylase: - The water and methanol extracts of both the plants did not show the inhibition of α-amylase enzyme up to a concentration of 10 mg/ml.

2.3.5.2 *In-vitro* α- glucosidase: The water and methanol extracts of both the plants did not show the inhibition of α- glucosidase enzyme up to a concentration of 10 mg/ml.

Chapter Three

Nutritional, Anti-nutritional component analysis, Isolation and partial characterization of starch from *Ariopsis peltata* and *Lagenandra toxicaria* tuber.

3.1 Introduction

The Araceae members are prominently used as human food as they are tuberous and rhizomatous filled with starch (Kuttigounder et al., 2011a; Singh et al., 2007). Starch is the basic source of nutrition and energy for plants and animals which is synthesized in plastids and stored in the form of semi-crystalline granules (Kuttigounder et al., 2011; Singh et al., 2007). Each plant starch has diverse physicochemical properties (Ochubiojo & Rodrigues, 2012) and most of the naturally occurring starches have limited application in the food industry owing to their poor process tolerance, high retrogradation, weak shear and heat resistance, higher

viscosity and thermal decomposition. Also, cooked starches form a weak, cohesive and rubbery paste (Alcázar-Alay & Meireles, 2015). Because of these limitations of conventionally available starch, investigators are looking for new sources of starch that have better physicochemical properties.

3.2 Methods

3.2.1 Nutritional analysis

The moisture content of freshly collected rhizomes was measured by drying them in a hot air oven at 100^0C until they reached a consistent weight. The technique specified by the Association of Official Analytical Chemists (AOAC, 1990) was used to determine the ash content. The percentage of the ash content was calculated. Crude fat was isolated by petroleum ether extraction at 60^0C for 8 hours using the soxhlet apparatus and the weight of crude fat was calculated (Lobo & Gulimane, 2015). Crude fibre content was extracted and the percentage of the crude fibre was calculated (Joslyn, 2012). The total amount of nitrogen was measured using macro Kjeldahl apparatus and crude protein content in the sample was calculated using the formula N x 6.25 (Balasubramanian & Sadasivam, 1987) The anthrone sulphuric acid technique was used to determine the total carbohydrate content (Sadasivam, 1996). The free amino acid profiling was done using a High-speed amino acid analyser L8900 (Sadasivam, 1996). The mineral content of the rhizome samples was measured as per the method of AOAC, 1990 and determined using atomic absorption spectroscopy (AAS). The nutritional value of the *Lagenandra toxicaria* and *Ariopsis peltata* rhizome sample was calculated by multiplying the protein, fat, and available carbohydrate values by 4, 9, and 4, respectively, and adding the results (Guil-Guerrero et al., 1998).

3.2.2 Antinutritional factors

The oxalate content of the rhizomes of both plants was determined using the titration method (Agbaire & Emoyan, 2012). Tannin content was determined using Follin Dennis spectrophotometric method.

3.2.3 Isolation of starch

The starch was isolated using sodium metabisulphite following the method of Chandra et al., (2016). The starch yield from each sample was calculated and starch content was determined using the anthrone method (Sadasivam, 1996). Apparent amylose content was determined

using the spectrometric method. Amylose content was calculated using potato amylose standard curve 100 mg/100 ml (Sadasivam, 1996). The water holding capacity of the starch was examined following Deepika et al., (2013). Paste clarity and percentage of transmittance was determined following the standard protocol (Ochubiojo & Rodrigues, 2012). The swelling property and solubility of the starch were observed and calculated at temperatures 60^0 C, 70^0 C, 80^0 C and 90^0 C (Chel-Guerrero et al., 2016). The shape and morphology were examined using a field emission scanning electron microscope (FESEM). Granule size was calculated using ocular micrometry (Lobo & Gulimane, 2015). The crystallographic pattern of the starch was determined using XRD [Rigaku miniflex 600 with CuKα radiation (λ= 0.15406 nm)]. The percentage of crystalline was measured in Origin pro 9.0 software (Malumba et al., 2017). The carbohydrate nature of the isolated starch sample was examined using ATR-FTIR (Ashwar et al., 2017). DSC-TGA (Sl.no 0600-1399 SDT Q600 V20.9 Build 20) was used to study the thermal property of the isolated starch (Abera et al., 2019). *In vitro* digestion of the starch was examined using the porcine pancreatic α-amylase (PPA) enzyme following Zhu et al., (2018).

3.3 Results

3.3.1 Nutritional analysis

The LT (*Lagenandra toxicaria*) showed a higher amount of moisture and ash content compared to the AP (*Ariopsis peltata*). The total carbohydrate content was significantly higher in the AP compared to LT. The protein content of LT and AP is 5.8% and 9.85% respectively. LT and AP have a low amount of crude fat content that is 1.29% and 0.89% respectively. The total calorific value of the AP is (216.7 % Kcal/100 g) higher when compared to LT (174.95 % Kcal/100 g). Both the plants have an anti-nutritional component like oxalate and tannin below the toxic level. AP and LT showed higher amounts of potassium, calcium and magnesium. LT contains more amounts of potassium, calcium and magnesium than AP. The microelements like iron, manganese and zinc content were higher in the LT than AP. Amino acid profiling of the rhizomes showed the presence of fourteen to sixteen different amino acids in the rhizome. The AP showed a higher amount of citrulline and g-ABA amino acids. The LT showed a higher amount of g-ABA, alanine and glutamine compared to other amino acids. LT has six essential amino acids among which lucien is higher and it also has two conditionally essential

amino acids. AP has five essential amino acid among which threonine is higher than other essential amino acids.

3.3.2 Starch

The isolated starch of the two different plant tubers was light brown and had a smooth texture. The starch yield and purity in AP were 24.72% and 99.26% respectively, while starch yield and purity in LT were 28.33% and 99.65% respectively. The pH of the isolated starch of LT is neutral while the starch of AP is slightly acidic. The apparent amylose content in LT and AP starch were 15.12% and 10.27% respectively. AP and LT starch show 357.8% and 314.18% of water holding capacity respectively. In the paste clarity, the percentage of transmittance increased in both samples. On the fifth day, LT showed 71.1% and AP showed 81.97 % transmittance. Swelling power and solubility increases with increasing temperature. AP and LT starch had 18.08% and 20.44 % of crystalline respectively and showed an A-type crystalline structure in the diffractogram. Chemical vibration authentication using ATR-FTIR confirms the carbohydrate nature of the starch. The granule size varied from 10 - 37.5 µm in LA starch and 5 - 25 µm in starch isolated from AP. The FESEM and optical microscopic images of the LT starch showed a smooth surface without any cleft and break. While the spherical shaped granules with both smooth and rough surfaces were seen in AP starch.

3.3.3 Thermal property

LT and AP showed higher gelatinization temperature and enthalpy. The weight loss of the starch of LT and AP occurred in the first section temperature range from 25^0 C to 240^0 C. The percentage of weight loss was 13.52 and 13.63 respectively. At 25^0 C to 240^0 C LT starch was slightly more stable than AP starch. In the second section of 240^0 C to 320^0 C weight loss of LT and AP starch was 71.30 % and 68.34% respectively.

3.3.4 *In vitro* digestion

The hydrolysis rate increased drastically for LT starch compared to the starch of AP in the first hours of the reaction. After one hour, the hydrolysis rate for AP starch increased when compared to LT starch. Both the samples showed a great extent of hydrolysis.

Chapter Four

Genotoxic and antigenotoxic potential of the *Lagenandra toxicaria* Dalz. and *Ariopsis peltata* rhizome methanol extracts using *Allium cepa* assay.

4.1 Introduction

The major storage protein in the members of Araceae is lectin (Van Damme et al., 1995) which is used in the development of anticancer therapeutics as it can bind to the cancer cell membranes and/or receptors causing the cell to undergo apoptosis. The *Lagenandra toxicaria* and *Ariopsis peltata* rhizomes have wide ranging therapeutic applications and it becomes imperative to ensure that the plant has genotoxic or antigenotoxic properties. The genotoxicity of plant products is an area of concern since it can covertly damage the genetic makeup. Genotoxic materials are also of interest since they can be potential candidates as therapies for diseases like cancer. The present study is to evaluate the genotoxic and antigenotoxic potential of *Lagenandra toxicaria* and *Ariopsis peltata* using *Allium cepa* root tip assay.

4.2 Methods

4.2.1 Genotoxicity test

Healthy onion bulbs were kept in dark for 3-4 days to allow the growth of fresh roots. The onion bulbs (n=6) with well developed roots were picked at their peak mitotic period and treated with LT and AP methanol extracts at concentrations 0.2, 0.5, 1, 5, and 10 mg/ml in 1% DMSO for 1, 2 and 4 hours. The 1% DMSO and water was used as controls, and the 3% H_2O_2 served as the positive control for genotoxicity. After the treatment, root tips were stained with aceto- orcein, squashed and observed under the microscope (Shetty et al., 2017). The cells were observed for mitotic aberrations in the randomly selected microscopic fields. About 700-800 cells were counted for each treatment and the MI and the cellular aberrations were examined. Root tips of each treatment (n=6) were dried in an oven for 15 hours at 60^{o}C and the dried samples were finely powdered. The powder was analyzed by FTIR (Rajeshwari et al., 2015). The root tips treated with 1% DMSO (control) and different concentrations of methanol extracts like 1 mg/ml, 5 mg/ml, and 10 mg/ml were excised, washed with PBS and observed

under FSEM at 5 kV voltage following the procedure of Ahmed et al., (2017) & Shetty et al., (2017). The extract treated roots were incubated in schiff's reagent for visualisation of the malondialdehyde which is a possible product of the lipid peroxidation (Chandel et al., 2017). The cell viability of the extract treated roots (n=6) was assessed using the 0.5% of 2, 3, 5-Triphenyl tetrazolium chloride (TTC) in phosphate buffer following the procedure of Sharma et al., (2018). Measurement of superoxide anion radical was carried out for the root tip (n=6) treated with 0.2, 0.5, 1, 5, and 10 mg/ml of the extract for four hours following the protocol Soltys et al., (2011). For the DNA integrity analysis, DNA was isolated from the treated root tip samples using Himedia plant genomic DNA Miniprep kit MB 507 - 20 PR, following the manufacturer's instructions. The gel pictures were documented using the UV CI-1100 ms major science system, Major science manufacturer, 19959 Sea Gull Way, Saratoga, U.S.A.

4.2.2 Antigenotoxicity test

The Antigenotoxic potential of plant extracts was examined using 3 % H_2O_2 induced clastogenicity. After being treated with 3 % H_2O_2 for 1 hour, roots were incubated with various plant extract doses (0.04, 0.02, 0.01, 0.005, and 0.0025 mg/ml) for 24 hours. The roots treated with the 3 % H_2O_2 served as the control. The squash was made and observed under the microscope. The antigenotoxicity potency of the plant extract was calculated. The group treated with one percent DMSO was taken as a negative control. The group treated with 3 % H_2O_2 and one percent DMSO was considered as a positive control. The lipid peroxidation of the treated root was evaluated using Schiff's reagent.

4.3 Results

4.3.1 Genotoxicity of the plant extract

There was a significant decrease in the root mitotic index (MI) in the roots treated with the LT and AP methanol extracts at the time points 1 hour, 2 hours and 4 hours in all concentrations used. The lowest dose of the extract used, i.e. 0.2 mg/ml brought a significant decrease ($p<0.05$) in the MI at 2 and 4 hours. But there was no significant effect upon its 1 hour treatment. The other concentrations such as 0.5 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml induced a significant decrease in the MI in a time and concentration dependent manner. The various cellular/chromosomal abnormalities such as chromosomal breaks, bridges, sticky metaphase, disturbed anaphase, multinucleated cells, lesions etc., were counted for each

treatment and expressed in percentage. Root surface ultrastructure after the four hour treatment with the extracts and the control was observed under the scanning electron microscope. The SEM images revealed a high degree of alterations in the surface morphology at the higher doses of both the plant extracts, root surface tissue shows fissures and cells were crumbled (5 mg/ml and 10 mg/ml) in comparison with the control. (20 µm and 10 µm scale). Both the extract treated and control samples were subjected to FTIR spectroscopy to examine the possible changes in the biochemical components of the roots. The obtained spectral peaks were assigned to their characteristic organic molecules and the changes in the peak positions due to the chemical shift were noted. The Peaks at the 1033 cm^{-1} and 1244 cm^{-1} were due to symmetric and asymmetric stretching of P=O. The 1545 cm^{-1} and 1633 cm^{-1} positioned peaks were because of the amide II and amide I of the beta sheets and alpha helix of the secondary structure of proteins respectively. 1735 cm^{-1} placed peak is due to ester C=O stretching. 2926 cm^{-1} placed peak is due to the C-H asymmetric stretch of the methyl group of lipids and a broad peak at 3302 cm^{-1} was assigned to the N-H stretching of proteins. The genomic DNA damage analysis was carried out to examine the possible mechanism of the plant extract induced genotoxicity. The isolated DNA from the negative control (1% DMSO), positive control (3 % H_2O_2) and 0.2, 0.5, 1, 5, and 10 mg/ml treated root samples for one hour and four hours were subjected to electrophoretic separation. An evident DNA damage was seen in the four hour treated roots at 5 mg/ml and 10 mg/ml. whereas only a slight DNA damage was seen in the roots treated with 10 mg/ml extract for one hour. The *in situ* histochemical localisation of the lipid peroxides were observed by Schiff's staining of the treated roots. Roots treated for four hours for all extract concentrations developed a prominent pink colour indicating the higher level of lipid peroxidation, whereas roots treated for one hour showed relatively less colour indicating the low level of lipid peroxidation. The accumulation of reactive oxygen species in the extract treated roots were visualised by NBT staining. A characteristic blue colour was observed in the four hour treatment which increased with the increase in the dosage of treatment. The cell viability of the plant extracts treated samples were checked and compared with the controls (normal control: water, negative Control: 1 % DMSO and positive control: 3 % H_2O_2). The one hour and two hours of treatment of the root tip with different concentrations of the extract showed no effect on the cell viability and were all stained pink as in the negative control. But the 10 mg/ml of *Lagenandra toxicaria* methanol extract treatment for 4 hours resulted in a moderate loss of cell viability showing less staining in comparison with the negative control.

4.3.2 The antigenotoxicity potential of the plant extract

The *Lagenandra toxicaria* plant extract showed a potential antigenotoxic activity and *Ariopsis peltata* did not show antigenotoxic activity at selected concentrations. The post treatment of the 3 % H_2O_2 treated roots with the 0.01 mg/ml and 0.02 mg/ml plant extract for 24 hours significantly decreased the clastogenic effects of the H_2O_2 treatment. The post treatment of 0.0025 mg/ml extract did not show any evident percentage inhibition and the higher concentration of 0.04 mg/ml treatment for 24 hours resulted in the total disruption of the root tip cells and hence they could not be counted. Schiff's reagent test revealed the colour intensity decreased in 0.01 mg/ml and 0.02 mg/ml post treated roots indicating the decreased lipid peroxidation. But the 0.04 mg/ml post treatment resulted in the enhanced pink colour indicating that at the higher concentrations the extract itself acts like a pro-oxidant.

Chapter Five (a)

Hepatoprotective potency of *Lagenandra toxicaria* and *Ariopsis peltata* against CCl_4 induced liver fibrosis in Wistar rats and identification of the bioactive compound.

5.1 Introduction

Liver is a major organ in our body that regulates various physiological functions. Repeated liver injury by toxicants, synthetic drugs, or hepatic viruses can cause sustained inflammation and wound healing processes that result in the accumulation of excess extracellular matrix (ECM) that does not undergo degradation leading to the fibrosis of the liver tissue (Guo et al., 2017; Hamza, 2010). The management of inflammation, oxidative damage, apoptosis, and related tissue responses are the essential therapeutic strategies for preventing liver failure (Lam

et al., 2016). The plant constituents have anti-inflammatory and antioxidant properties that can have multiple effects in counteracting liver fibrosis and recovering the damaged cells (El-Tantawy & Temraz, 2019). It is crucial that ethnomedicinally beneficial plant's efficacy toward liver therapy has to be scientifically evaluated. The present study examined the anti-inflammatory and hepatoprotective activities of the rhizome extracts of LT and AP.

5.2 Methods

In-vitro anti-inflammatory study was carried out by human red blood cells (HRBC) membrane stabilization method as given by (Vogel, 2002). Acute oral toxicity study was performed as per OECD guidelines for the testing of chemicals, Test No. 423 (OECD, 2001; Acute oral toxicity – acute toxic class method) using *Mus musculus* female mice of 8-10 weeks of age weighing 25-30 g. The hepatoprotective activity of the plant extracts was studied using CCl_4 Induced liver fibrosis in the male Wistar rats. The experimental design involved 12 groups containing n= 8 in each group. The animal experiments were approved by the animal ethical committee of Nitte institute, deemed to be University, the protocol number (NGSMIPS /IAEC /MARCH-2019/ 124).

Group 1: Normal control + saline
Group 2: Positive control (CCl_4 + Olive oil (1:1, 1 ml/kg B. w) twice a week (t. w))
Group 3: Negative control (CCl_4 + 0.1 % DMSO)
Group 4: CCl_4 (t. w) + Silymarin 100 mg/kg
Group 5: CCl_4 (t. w) + *Lagenandra toxicaria* methanol extract 600 mg/kg
Group 6: CCl_4 (t. w) + *Lagenandra toxicaria* methanol extract 400 mg/kg
Group 7: CCl_4 (t. w) + *Lagenandra toxicaria* water extract 600 mg/kg
Group 8: CCl_4 (t. w) + *Lagenandra toxicaria* water extract 400 mg/kg
Group 9: CCl_4 (t. w) + *Ariopsis peltata* methanol extract 600 mg/kg
Group 10: CCl_4 (t. w) + *Ariopsis peltata* methanol extract 400 mg/kg
Group 11: CCl_4 (t. w) + *Ariopsis peltata* water extract 600 mg/kg
Group 12: CCl_4 (t. w) + *Ariopsis peltata* water extract 400 mg/kg

The CCl_4 was given twice a week at an interval of 3 days. The standard drug silymarin and the plant extracts were administered every day for 8 weeks (Vogel, 2002). After eight weeks of treatment, the animals were sacrificed by cervical dislocation. The blood samples were collected and used for serum enzyme analysis. The liver tissues were isolated and a portion was

stored in formalin for histopathological studies. The remaining tissues were homogenized (10 %, w/v) in 50 mM phosphate buffer (pH. 7.0) and stored at -80^0 C for analysis. The body weight and liver weight of each animal were recorded and the liver index of the rats was calculated (Wang et al., 2018). The levels of serum AST, ALT and total bilirubin activities were examined by Agappe kits (India) using semi auto-analyzer (STAR 21) following the manufacturer's instructions. For histological observation of liver damage, a part of the liver tissue was fixed and stained with hematoxylin-eosin (H&E) and collagen deposition in the liver was observed using Masson's trichrome stain (Jayakumar et al., 2006). The presence of necrosis, inflammation and fatty metamorphosis in the histological observation was assessed using a 0-3 scoring system (grade 0-absent, grade 1-present in one-third of the lobule, grade 2-present in two-thirds of the lobules, grade 3-present in all the lobules). For fibrosis, collagen was graded according to Gui et al., (2006) grade 0-normal, grade 1-fibrosis present (collagen fibre present as small septa) grade 2-mild fibrosis (collagen fibre extended as septa from portal tract to central vein forming complete septa); grade 3-moderate fibrosis (collagen fibres formed thin complete septa) and grade 4-severe fibrosis (collagen fibers formed thick septa). All histological assessments were carried out by a skilled operator who was blinded to the study. Liver homogenate supernatant was used to determine the MDA levels and catalase activity of the liver following the methods of Sinha (1972). Bioactive compounds present in *Lagenandra toxicaria* and *Ariopsis peltata* rhizome methanol extract was analyzed using GC-MS instrument, Shimadzu QP-2010 ultra. The identities of the bioactive compounds were done using the Willey/ NIST mass spectral library of the GC-MS data system.

5.3 Results

5.3.1 Anti-inflammatory activity

The anti-inflammatory activity of the LT and AP methanol and water extracts was analyzed using the human red blood cells. Both the plant extracts showed significant stabilization of HRBC at different concentrations (200 mg/ml, 400 mg/ml, 600 mg/ml and 800 mg/ml). The *Lagenandra toxicaria* methanol extract showed more protection compared to all other extracts.

5.3.2 Acute toxicity study

The acute toxicity studies showed that the *Lagenandra toxicaria* and *Ariopsis peltata* rhizome methanol and water extract at 2000 mg/kg dose did not show any behavioural changes or mortality in treated animals.

5.3.3 Bodyweight and liver index

In the positive control group and negative control group bodyweight decreased significantly ($p<0.05$) and mortality was observed in two of the animals compared to the normal control group. A significant increase in body weight was seen in the *Lagenandra toxicaria* treatment groups. The liver weight and liver index were significantly enhanced in the positive control group compared to the normal control group. The increased liver weight and index in the positive control group were again seen to be decreased significantly in the ($p<0.05$) treatment groups

5.3.4 Serum markers of CCl_4 induced liver fibrosis in animals

The concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TBL) levels in the serum of different treatment groups when compared to the normal control group and there was a significant ($p<0.05$) increase in the enzyme levels in the positive and negative control groups. Among the extract-treated groups, the LM extract at 600 mg/kg B. w. showed a better outcome where the levels of AST, ALT, and TBL decreased significantly ($p<0.05$) compared to the positive control group and reached near normal.

5.3.5 Liver histology

The effect of the extract on CCl_4 induced liver fibrosis was determined by H&E staining and Massonson trichrome staining. The H&E and Massonson trichrome staining revealed that the CCl_4 treated animal liver showed prominent hepatic steatosis, necrosis, infiltration of inflammatory cells, hepatocyte disorganization, sinusoidal architectural changes and collagen deposition confirming the successful induction of liver fibrosis. The pathological effects of CCl_4 treatment were seen to be ameliorated by the plant extracts. 600 mg/kg *Lagenandra toxicaria* methanol treatment reduced the CCl_4 induced liver necrosis, inflammation and collagen deposition. Histological scoring also indicates a significant reduction of severity of inflammation, fatty metamorphosis, necrosis and collagen deposition compared with the positive control group.

5.3.6 Liver antioxidant enzyme and oxidative stress marker

The positive control group animal liver homogenate showed a decreased catalase activity and a high level of MDA compared to the normal group. In contrast, homogenate from the plant extract-treated group showed significantly lower levels of MDA and significantly increased

levels of catalase activity compared to the positive control group. The plant extract of LM at 600 mg/kg treated group showed a similar outcome as the standard drug.

5.3.7 GC-MS analysis

The chromatogram revealed the presence of 21 compounds in *L. toxicaria* and 51 compounds in *A. peltata* methanol extract. The chromatogram of the LT extract showed a good amount of 9-octadecenamide with a peak area of 36.53 % and the chromatogram of AP extract showed higher amount of palmitic acid or hexadecanoic acid with a peak area of 13 %.

Chapter Five (b)

Separation and characterisation of phytochemicals from *Lagenandra toxicaria* methanol extract and their antioxidant, anti-inflammatory and hepatoprotective studies.

6.1 Methods

The fractionation of the *Lagenandra toxicaria* methanol extract was done using solvent-solvent fractionation based on the polarity. The antioxidant properties of the fractions were assessed using DPPH radical scavenging assay, and the IC 50 of the fractions was calculated following the formula given by Sethi et al., (2020). HRBC membrane stabilisation method was used to test the fractions' anti-inflammatory activity using different concentrations like 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml (Saleem et al., 2011). Acute oral toxicity study was performed as per OECD guidelines for the testing of chemicals, Test No. 423 (OECD, 2001; Acute oral toxicity – acute toxic class method). Induction of liver fibrosis was done using CCl_4. The experimental design involved 12 groups containing n= 8 in each group.

Group 1: Normal control + saline

Group 2: Positive control (CCl_4 + Olive oil (1:1, 1 ml/kg B. w.) twice a week (t. w))

Group 3: Negative control (CCl_4 + 0.1 % DMSO)

Group 4: CCl_4 (t. w) + Silymarin (100 mg/kg, everyday up to 8 weeks).

Group 5: CCl_4 (t. w) + fraction FR-1 60 mg/kg

Group 6: CCl_4 (t. w) + fraction FR-2 60 mg/kg

Group 7: CCl_4 (t. w) + fraction FR-3a 60 mg/kg

Group 8: CCl_4 (t. w) + *fraction FR*-3b 60 mg/kg

Group 9: CCl_4 (t. w) + fraction FR-3c 60 mg/kg

The body weight and liver weight of each animal were recorded and the liver index of the rats was calculated following the formula given by Wang et al., (2018). The levels of serum AST, ALT and total bilirubin activities were examined by Agappe kits (India) using semi auto-analyzer (STAR 21) following the manufacturer's instructions. Liver histology examination was done. The liver homogenate supernatant was collected to determine the MDA levels and catalase activity of the liver was calculated following Sinha (1972). Bioactive compounds present in FR-3a fraction was analyzed using the GC-MS instrument, Shimadzu QP-2010 ultra. The 1H NMR analysis of fraction (Fr-3a) was done to confirm the presence of the bioactive compound.

6.2 Results

6.2.1 Fractionation

The solvent-solvent fractionation of the *Lagenandra toxicaria* rhizome methanol extract was done. Yield of each fraction was calculated. In comparison to the other fractions, the yield in the petroleum ether fraction (FR-1) was relatively low, and the yield in the methanol fraction (FR-3) was more. In the methanol sub-fractionation, 10 % formic acid (FR-3c) yielded the least, while methanol and water mixture (FR-3b) yielded the most when compared to the ethyl acetate and methanol mixture (FR-3a).

6.2.2 DPPH radical scavenging activity assay

The IC 50 value of the fraction FR-3a was 369.448 µg/ml is lower compared to the IC 50 of the other fractions of *Lagenandra toxicaria* methanol extract.

6.2.3 Anti-inflammatory activity

In the anti-inflammatory activity stabilization of HRBC at different concentration were examined (20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml). The FR-3a showed more protection compared to all other fractions. At the concentration of 40 mg/ml and 60 mg/ml, it showed 31.19±6.09 % and 41.99±3.32 % of protection respectively against hemolysis. There was less protection at higher concentrations of extract/fraction.

6.2.4 Acute toxicity study

The acute toxicity tests revealed that there are no behavioural abnormalities or death in extract/fraction of *Lagenandra toxicaria* treated animals at 2000 mg/kg. As a result, the extract/fractions were considered nontoxic.

6.2.5 Body weight and liver index

When compared to the normal control group, bodyweight in the positive and negative control groups significantly ($p<0.05$) decreased and mortality was recorded in two of the animals. Fractions and silymarin treated group did not show any mortality except Fr-1 and FR-3c treatment group. The bodyweight of all the animals significantly increased in the treatment groups compared to the positive group. The liver weight and liver index were significantly higher in the positive group compared to the normal control group. The increased liver weight and index in the positive group were again seen to be decreased significantly in the ($p<0.05$) FR-3a and FR-2 treatment groups.

6.2.6 Serum markers of CCl_4 induced liver fibrosis in animals

AST, ALT, and TBL levels in the blood are strong markers of liver health, and a rise in these enzyme levels suggests aberrant liver function. In this study, positive control groups that received CCl_4 alone had higher levels of AST, ALT, and TBL than the other groups, showing that CCl_4 caused liver damage. The CCl_4 treated group had a significant ($p<0.05$) increase in enzyme levels compared to the normal control group, the fraction treated group FR-3a showed promising results compared to the positive control group, the levels of AST, ALT, and TBL were considerably reduced ($p<0.05$) and returned to near-normal levels.

6.2.7 Liver histology

The harmful consequences of CCl_4 were observed to be considerably reduced by FR-3a 60 mg/kg B. w. treatment group compared to the other fraction treated group, which reduced CCl_4 - induced liver necrosis inflammation and collagen deposition. When compared to the positive control group, histological scoring of the FR-3a treated group also shows a significant reduction in the degree of inflammation, fatty metamorphosis, necrosis and also severity of the collagen deposition. The FR-3a at 60 mg/kg B. w treated group showed a similar outcome as the standard drug treated group.

6.2.8 Liver antioxidant enzyme and oxidative stress marker

The positive control group showed low catalase activity and high level of MDA, a lipid peroxidation marker in the liver homogenate compared to the normal group. In contrast, the FR-2 and FR-3a treated group liver homogenates showed significantly lower levels of MDA and significantly increased levels of catalase activity compared to the positive control group. The FR-3a at 60 mg/kg B. w treated group showed a similar outcome as the standard drug treated group.

6.2.9 GC-MS analysis of the FR-3a

GC-MS analysis of the FR-3a showed the presence of 9-octadecenamide with an 81 % larger peak area and isoamyl acetate with an 11% peak area.

6.2.10 ^{1}H NMR analysis of FR-3a

In the proton NMR (CDCl3, 400 MHZ) spectrum of 9(z)-Octadecenamide, the characteristic absorption appeared at δ5.347-5.334 (2H, m, -CH=CH-), 2.348-2.311(2H, t, J=7.4 Hz -CH_2-CO-NH_2), 2.065-2.000 (2H, m, -CH_2-CH=CH-CH_2), 1.618-1.601 (4H, m, CH2-CH2-CO-NH2), 1.293-1.246 (20H, m, 10 CH2), 0.871(3H, t, J=6.6 Hz CH_3).

Summary and conclusion

The phytochemical analysis of the *Lagenandra toxicaria* and *Ariopsis peltata* plants showedpresence of phenol, flavonoids, steroids, saponin and tannin. The quantitative analysis confirmed that the LT methanol extracts contained more phenolic and flavonoid components than the other LT and AP extracts. Additionally, LT methanol extracts had stronger antioxidant activity than LT water, AP methanol and water extracts. In the nutritional and anti-nutritional analysis both the plant had higher contents of carbohydrates and minerals. Oxalate and tannin contents were found to be below toxic level. Both the plants have 5-6 essential amino acids. The starch yield in both the tubers was significant. Both the aroid starch had low amylose content and small granule size with an A-type crystalline structure. The starch from both the tubers contains low amylose content which renders it suitable as a food thickening agent.

In the genotoxicity and anti-genotoxicity study revealed that the *Lagenandra toxicaria* and *Ariopsis peltata* rhizome methanol extract possesses genotoxic property at higher concentration and *Lagenandra toxicaria* rhizome methanol extract also shows anti-genotoxic properties at lower concentration. The extract induces genotoxicity at higher doses at higher time intervals which were confirmed by the decreased MI, increased ROS generation, DNA damage and lipid peroxidation. Also in lower concentration the LM extract protects the *Allium cepa* cells against 3% H_2O_2 induced genotoxicity. These results could be helpfull for the development of cancer therapetics.

In the HRBC membrane stability assay, the methanol extract of *L. toxicaria* exhibits a greater anti-inflammatory capacity than the other extracts. Both the plant extracts showed resistance to CCl_4-induced liver fibrosis. The methanol extract of *L. toxicaria* 600 mg/kg showed higher amelioration of CCl_4 induced collagen accumulation and inflammation. The GC-MS analysis of *L. toxicaria* methanol extract revealed the presence of bioactive compounds such as octadecenamide, palmitic acid and stigma steroids, which have anti-inflammatory and antifibrotic activities. Four major fractions and three sub-fractions were separated during the purification and characterization of phytochemicals from LM extract. Among the other fractions FR-3a fraction demonstrated remarkable anti-fibrotic activity against CCl_4 induced liver fibrosis. The *In-vitro* study results proved the anti-inflammatory and antioxidant nature of the FR-3a fraction. The same fraction's GC-MS analysis revealed that the peak area for 9-octadecenamide was higher**.** The proton NMR and confirms the presence of the 9-octadecenamide (oleamide). Study proved that oleamide suppress the NF-κb activity and also

reduces inflammation. This may be one of the mechanisms where oleamide suppresses liver fibrosis.

The findings like antioxidant, antifibrotic, antigenotoxic and anti-inflammatory potency of the plant also its nutritional components and its bioactive compounds support the use of this plant in traditional remedies and nutritional purpose. The starches can be used as a substitute for simple sugars in the brewing and pharmaceutical industries as they mimic simple sugar in digestibility. Studies have also prompted a new interest in oleamide's antifibrotic properties.

List of publication

1. Akarsha B., & Krishnakumar G. (2021). Genotoxic and antigenotoxic potential of the *Lagenandra toxicaria* dalz. rhizome methanol extract using *Allium cepa* assay. *Asian Journal of Pharmaceutical and Clinical Research, 14*(5), 82–90. (ISSN 0974 - 2441).

2. Akarsha B, Krishnakumar G, Prasanna Shama Khandige (2022). Hepatoprotective potency of *Lagenandra toxicaria* and *Ariopsis peltata* against CCl_4 induced liver fibrosis in Wistar rats. *Plant science today* (eISSN: 2348-1900) **(Accepted).**

3. Akarsha B, Karunya Shetty, Krishnakumar G (2022). Nutritional, Anti-nutritional component analysis, Isolation and partial characterization of starch from *Ariopsis peltata* and *Lagenandra toxicaria* tuber. Helyion (**Communicated and reviewed but decision is pending)**.

List of papers presented at conferences.

1. Akarsha B & Krishnakumar G. Antioxidant potency of *Lagenandra toxicaria* and *Ariopsis peltata.* International conference on synergy of sciences (ICSS - 2020). School of Chemical and Biotechnology SASTRA Deemed University, Thanjavur, Tamilnadu. 27^{th} -28^{th} February, 2020.

2. Akarsha B & Krishnakumar G. Genotoxic effect of *Ariopsis peltata* rhizome methanol extract using *Allium cepa* assay. International conference on new horizon in plant science (NHPS 2020). Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, Kerala, India. 4^{th} - 9^{th} January 2021. 99-100. (ISBN 978-81-940888-3-7).

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